Bioorthogonal antigens as tool for investigation of antigen processing and presentation

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Summary

This thesis describes research on the use of bioorthogonal protein as antigens in immunological studies. Bioorthogonal proteins are defined as proteins in which one of the canonical amino acids is replaced by a non-canonical one, the side-chain of which carries a bioorthogonal functionality - a functionality that is inert in physiological samples (at ambient temperature, pressure, neutral pH and aqueous conditions) and that can be made to react effectively and selectively to bring added functionality at a desired time during the course of a biological experiment. In the context of the research described in this thesis, bioorthogonal proteins are created and utilised in which methionine residues in the protein backbone are replaced by azidohomoalanine or homopropargylglycine - biological methionine isosteres that are small (thus are not likely to perturb protein functionality much), are physiologically largely inert, yet are reactive in the copper-catalysed Huisgen reaction, or - in case of azidohomoalanine - in the Staudinger and strain-promoted azide-alkyne cycloaddition reactions as well, and can be readily introduced in recombinant proteins by utilising methionine auxotroph bacterial expression strains.

Main focus of this thesis research has been to establish procedures that make use of bioorthogonal proteins to study proteolysis events that take place in dendritic cells during antigen presentation. Factors that partake in antigen processing such as dynamics, hierarchy of proteolytic events (which proteases are involved and in what order) and in which subcellular compartments are currently very hard to study. Simply incubating antigen-presenting cells with antigenic peptides equipped with reporter molecules fails to give a good picture of what happens in natural antigen-processing because antigen trafficking and proteolysis are intrinsically linked. The rate of proteolysis is highly dependent on the precise sequence and structure of the protein, which hampers or even obviates the use of engineered/modified antigenic proteins bearing (large, often hydrophobic) fluorophores as reporter entities (this thesis Chapter 2). A final complication comprises the harsh chemical conditions found within the antigen presenting pathway of the dendritic cell. On its way to being major histocompatibility complex (MHC)-surface loaded, the peptide will encounter oxidative as well as reducing conditions, besides the acidic environment as found within lysosomes - conditions that fluorophores may not withstand (and incidentally also precludes the use of alternative bioorthogonal tags such as trans-cyclooctenes). For these reasons, antigenic proteins containing either azidohomoalanine or homopropargylglycine as non-canonical amino acids are put forward here for a variety of studies in the context of antigen processing and presentation. The research described in the consecutive chapters of this thesis covers both the generation of such bioorthogonal antigens and the study of their fate in a number of *in vitro* and *in situ* proteolysis studies.

Chapter 2 introduces antigen processing and presentation as a central pathway in adaptive immunity in vertebrates, the techniques that are available to study processes involved in these, and bioorthogonal chemistry, its scope and limitations and its potential to aid in the study of antigen processing and presentation, the latter as indicated in Chapter 1 in which this thesis work is introduced.

Chapter 3 describes the expression of bioorthogonal variants of the model antigen, ovalbumin in a methionine auxotrophic *Escherichia coli* strain. Replacing methionine with azidohomoalanine or homopropargylglycine during the expression yielded the antigen carrying 17 reactive groups per protein. These proteins were then assessed in their ability to activate cognate T cells after processing by dendritic cells, proving to be near identical in their antigenic properties to the wildtype of the antigen. In addition, it was shown that the degradation of the antigen could be followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the fate of the antigen tracked by confocal microscopy. These data combined suggest bioorthogonal antigens to be suitable reagents for studying antigen processing.

Chapter 4 describes the detailed study of the proteolysis of the bioorthogonal antigenic proteins that were prepared in Chapter 3. By assessing the cleavage rates by different recombinant proteases *in vitro*, the effects of the bioorthogonal modification on this degradation can be studied in detail. These experiments show that this fine specificity changes < 20 % upon introduction of the chemically reactive groups. It was also analysed whether the post-translational modification - carbamylation and citrullination - of the proteins did lead to altered processing. This was indeed observed: carbamylation in particular led to significant reductions in the rates of proteolysis for various endo-lysosomal proteases. Interestingly, this was also true for the auto-antigen vinculin of which post-translational citrullination and carbamylation have been implicated in the initiation of the pathogenesis of rheumatoid arthritis.

This led to the research described in **Chapter 5**, in which the altered antigenicity of these proteins is described, confirming that bioorthogonal antigens were useful reagents for studying the processing of these modified proteins.

Chapter 6 summarises the results of this work and shows possible perspectives that can be used for continuation of this research.